2019 Project Abstract
For the Period Ending June 30, 2023

PROJECT TITLE: Stimulating bacteria to degrade chlorinated industrial contaminants
PROJECT MANAGER: Paige J. Novak
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2019, First Special Session, Chp. 4, Art. 2, Sec. 2, Subd. 04s as extended by M.L. 2022, Chp. 94, Sec. 2, Subd. 19 (c.1) [to June 30, 2023]

APPROPRIATION AMOUNT: $ 150,000
AMOUNT SPENT: $ 150,000
AMOUNT REMAINING: $ 0

Sound bite of Project Outcomes and Results
A group of bacteria exist that can "breathe" chlorinated pollutants. Naturally occurring chlorinated compounds are formed when leaves and pine needles break down. We discovered that these naturally occurring compounds can speed the rate at which chlorinated pollutants are degraded when added as an amendment.

Overall Project Outcome and Results
Over half of the contaminated sites in Minnesota contain chlorinated pollutants—chemicals that contain chlorine atoms attached to a carbon framework—that can cause serious health effects. Once the chlorine atoms are removed, the carbon framework is non-toxic. Bacteria exist that can remove the chlorine atom from this carbon framework. One kind of bacteria "breathes" these compounds by removing the chlorine atom from the carbon framework (so-called halorespiring bacteria (HB)). As a result, they require the presence of chlorinated compounds to survive. Other bacteria (non-respiratory dechlorinators (NRD)) remove the chlorine atom to use the carbon framework for growth. Little is known about them. Natural chlorinated compounds (not pollutants) also exist as a natural part of soil (called chlorinated natural organic matter, or Cl-NOM).

In this research project the goals were to determine whether and how Cl-NOM stimulated dechlorination of pollutants. We found that Cl-NOM addition to HB led to the faster and more complete dechlorination of the common pollutant perchloroethylene (PCE). Currently, engineers actually add similar bacteria to the ones we tested to contaminated sites to improve degradation. Our research suggested that to further improve the degradation of these pollutants, Cl-NOM could be added with HB to increase the rate (and possibly the extent) of chlorine removal, reducing the time and cost to reach clean-up goals. We found that NRD are widespread and can dechlorinate chlorinated contaminants and grow as a result, potentially giving them a competitive advantage in nutrient-limited environments. Nevertheless, though Cl-NOM amendment selected for some HB and NRD, these organisms did not necessarily have the ability to dechlorinate the most common pollutants. One graduate student focused on this research for her Master's thesis (submitted to LCCMR) and another postdoctoral researcher published one peer-reviewed manuscript supported in part by this research (also submitted to LCCMR).

Project Results Use and Dissemination
As stated above, one peer-reviewed manuscript was published from this work and has been submitted to the LCCMR. Multiple presentations about the research have been given at conferences. The student supported by this funding for her Master's research is now a practicing engineer for a large regional firm, and as such is able to further disseminate these results.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2019 ENRTF Work Plan (Main Document)

Date of Submission:	August 7, 2023
Final Report	
Date of Work Plan Approval:	June, 5, 2019
Project Completion Date:	June 30, 2023

PROJECT TITLE:	Stimulating bacteria to degrade chlorinated industrial contaminants
Project Manager:	Paige J. Novak
Organization:	University of Minnesota
College/Department/Division:	Department of Civil, Environmental, and Geo- Engineering
Mailing Address:	122 Civil Engineering Building, 500 Pillsbury Drive SE
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Location: Statewide

Total Project Budget:	$150,000
Amount Spent:	$150,000
Balance:	$0

Legal Citation: M.L. 2019, First Special Session, Chp. 4, Art. 2, Sec. 2, Subd. 04s as extended by M.L. 2022, Chp. 94, Sec. 2, Subd. 19 (c.1) [to June 30, 2023]

Appropriation Language: $1,000 the first year is from the trust fund to the Board of Regents of the University of Minnesota to determine the best way to stimulate bacteria to more quickly and completely remove industrial chlorinated pollutants from contaminated sites. On the day following final enactment, the following amounts from unobligated appropriations to the Board of Regents of the University of Minnesota are transferred and added to this appropriation: $75,000 in Laws 2016, chapter 186, section 2, subdivision 4, paragraph (l), and $74,000 in Laws 2016, chapter 186, section 2, subdivision 6, paragraph (b).

M.L. 2022 - Sec. 2. ENVIRONMENT AND NATURAL RESOURCES TRUST FUND; EXTENSIONS. [to June 30, 2023]

I. PROJECT STATEMENT:

Minnesota contains a large number of contaminated sites that require clean-up at a large cost. Indeed, according to the Minnesota Pollution Control Agency's most recent report, there are 92 contaminated sites on the Minnesota "Superfund" List. These sites are either abandoned or the contamination is uncontrolled, causing concern. At these sites alone, $13,500,000 was spent in FY 2015-16 on clean-up tasks. In addition to these Superfund sites, there are 621 additional contaminated sites in Minnesota that currently require clean-up. Over half of the contaminated sites in Minnesota contain chlorinated pollutants that are known or suspected to cause serious human health effects. Research is needed to develop ways to affordably clean up chlorinated pollutants, safeguarding current and future human and economic health.

Interestingly, bacteria exist that can "breathe" toxic chlorinated pollutants (so-called halorespiring bacteria). To survive, however, they require the presence of chlorinated pollutants. As a result, higher concentrations of chlorinated pollutants typically sustain these organisms more effectively. Nevertheless, during remediation, we want to remove or degrade chlorinated pollutants to very low concentrations, which can make it difficult to sustain these halorespiring bacteria. If these bacteria are being used to clean-up a site containing chlorinated pollutants, the result can be a "stalling" of the process at concentrations of pollutant that are too low to sustain the halorespiring bacteria, but too high to be protective of human and ecological health.

Natural chlorinated compounds (not pollutants) also exist in low concentrations in uncontaminated sites as a natural part of soil. In our research, we have found that these natural chlorinated compounds can stimulate pollutant dechlorination in both halorespiring bacteria and other bacteria that use the dechlorinated carbon for growth, called "non-respiratory dechlorinators." We suspect that these non-respiratory dechlorinators are able to dechlorinate pollutants to lower concentrations because, while they can use them and therefore degrade them, those bacteria do not rely solely on the chlorinated pollutants to survive.

We hypothesize that amendments with different amounts of soil-based carbon versus natural (non-pollutant) chlorinated compounds will stimulate halorespiring bacteria and non-respiratory dechlorinators differently. This can be used to verify that non-respiratory dechlorinators can dechlorinate pollutants to desired low concentrations and enable the addition of amendments to control the rate and extent of pollutant dechlorination based on the amount of pollutant present. In the proposed research we will test this hypothesis with the goal of determining the best way to stimulate both groups of bacteria with natural compounds for pollutant dechlorination to low concentrations, saving money and time, and reducing risk.

II. OVERALL PROJECT STATUS UPDATES:

First Update March 1, 2020

Project activities over the last seven months have included some method development, acquisition of contaminated aquifer material with which to begin experiments, and construction of an anaerobic continuous flow reactor designed to culture dechlorinating bacteria with constant exposure to either NOM or Cl-NOM. The reactor is currently being started with Cl-NOM supply and will be monitored over time for evidence of dechlorination and for growth of dechlorinating bacteria and increases in the number of genes present that code for dechlorinating enzymes. In the first project period the recruitment and hiring of a graduate research assistant has also taken place. The student working on the project has a background in environmental consulting and is well-suited to the research. Nevertheless, she is currently part time. Therefore, the majority of the experiments will start this summer.

Second Update September 1, 2020

Project activities over the last seven months included development of methods and training of the new student to work on the project. The continuous flow reactor was also fed Cl-NOM and dechlorination was monitored over time and samples were taken for later evaluation of the genes and bacteria present.

Laboratories at the University of Minnesota were shut down as a result of the COVID-19 pandemic from March 16, 2020 to May 11, 2020. Before returning to the lab, the student working on the project had to undergo training, which further delayed re-entry to the laboratory as students that required training could not immediately return to the laboratory. The student worked on a review of the literature during this period, which will be valuable to the project and will make the work go more rapidly and seamlessly in the future. The student began training and started supervised lab work in July of 2020 and began independent lab work in August 2020. Since August, she has been preparing her materials to start the dechlorination experiments. Initial dechlorination experiments have now begun.

Third Update March 1, 2021

Project activities over the last six months include the continuous monitoring of the flow-through reactor, DNA extraction and sequencing, and the set-up of several smaller-scale experiments in preparation for a larger experimental run. The anaerobic continuous flow reactor is being monitored weekly for dechlorination activity with biomass samples also taken to monitor for the presence of genes linked to dechlorination. DNA was extracted from the biomass samples taken during the first few months of reactor operation and were submitted for sequencing. Sequencing results are expected in the next few weeks, after which they will need to be analyzed to determine how the diversity of the microbial community and abundance of specific reductive dechlorinating bacteria change in the reactor over time and as a function of dechlorination activity. The student working on the project has been undergoing training for such analysis. In addition, several small-scale dechlorination experiments have been set-up to determine how best to set up and run these experiments at a larger scale.

Fourth Update September 1, 2021

We have struggled in the last project period with establishing a robust PCE-dechlorinating culture. This culture is important to have so that we can amend it with natural organic matter (NOM) and chlorinated natural organic matter (Cl-NOM) and observe the resulting impacts on PCE dechlorination. We have been in contact with several colleagues, and one has agreed to send a dechlorinating culture that we can use in our experiments. These experiments will begin once the culture arrives. In preparation for these experiments, the student working on the project has extracted NOM from several soil samples and has chlorinated a portion of the NOM (Cl-NOM) for addition to the dechlorinating experiments. The student has also been continuing to operate the flow-through reactor, adding higher quantities of Cl-NOM to the reactor so that she can monitor the microbial response. Samples have been taken from the reactor and analyzed for chloride to assess Cl-NOM dechlorination. Samples have also been taken for biomass analysis and the DNA from these samples has been extracted. The student has been analyzing the DNA sequencing data generated to date. Finally, the student on the project has ordered the necessary supplies to quantitatively assess the various organisms present as well as the dechlorinating genes present in the flow through reactor over time. These same supplies will be used to monitor the populations and genes in the PCE-dechlorinating culture upon addition of NOM and Cl-NOM.

Amendment Request (12/03/2021):

This formal amendment request is to seek approval for the addition of undergraduate researchers in the "Personnel" category of the budget. We anticipate that approximately $5,000 of the currently budgeted personnel funds will be used for an undergraduate researcher. The graduate student working on the project was not full time for several semesters; as a result, a small amount of funding is available to pay an undergraduate research assistant to help the graduate student analyze some of her samples, allowing more of her time to be spent on bioinformatic data analysis. This change will not impact the movement of funds between budget categories and will not impact the project outcomes or time-line.

Amendment approved by LCCMR 12/10/2021

Fifth Update March 1, 2022

The student received the dechlorinating culture in November of 2021 from a colleague and it has been maintained with specific media and PCE addition. With this culture, 100 µM of PCE was degraded in less than 2

weeks to daughter products TCE and DCE. DNA was collected from different time points of this experiment and will be extracted. Once extracted, analyses will be performed to determine how the numbers of the specific dechlorinating bacteria present changed over time. The experiment is currently being repeated with the addition of chlorinated NOM and NOM to determine the impact of those additions on PCE dechlorination. Initial data suggested that the PCE degraded very rapidly in the presence of chlorinated NOM and NOM. Again, the populations that changed in number over the degradation process will be determined.

The flow-through reactor was decommissioned in February 2022, after more than 1 year of biomass and chloride data collection. Samples from the flow-through reactor have been collected weekly for biomass analysis as well as chloride concentration analysis. The DNA from that biomass has been extracted and either sequenced or sent off for sequencing at the University of Minnesota Genomics Center. These data are being analyzed to determine which bacteria and dechlorinating genes are present and correlate with operational periods during which high concentrations of chlorinated NOM were fed or the chlorinated NOM was effectively dechlorinated. This will indicate whether the dechlorination of the NOM was a respiratory or non-respiratory process.

Amendment Request (2/22/2022):
This formal amendment request is to seek approval for the movement of $12,000 from the "Equipment/Tools/Supplies" category to the "Personnel" category. As a result of the pandemic and its impact on the ability of the graduate and undergraduate students to work consistently over the grant period, we anticipate needing to shift funds to support the current graduate student through the end of the project. This change will not impact the project outcomes or time-line.

Amendment approved by LCCMR 2/24/2022

Update as of June 30, 2022
Project extended to June 30, 2023 by LCCMR 6/30/22 as a result of M.L. 2022, Chp.94, Sec. 2, Subd. 19, legislative extension criteria being met.

Sixth Update September 1, 2022
Experiments were performed to monitor the PCE dechlorination of obligate halorespiring bacteria (HB) in the absence of NOM and Cl-NOM ("no Cl-NOM"), in the presence of high concentrations of NOM plus low concentrations of Cl-NOM ("low Cl-NOM"), and in the presence of low concentrations of NOM plus high concentrations of Cl-NOM ("high Cl-NOM"). Results showed that when obligate HB are amended with Cl-NOM, dechlorination of PCE is faster and formation of the PCE daughter product, *cis*-DCE, occurs. When amended with a higher concentration of Cl-NOM, PCE dechlorination is faster compared to when cultures are amended with low concentrations of Cl-NOM. In the no Cl-NOM reactors, no *cis*-DCE was detected, suggesting that dechlorination was less complete. These results suggest that addition of Cl-NOM at higher volumes to known obligate HB should be beneficial to dechlorination.

There are multiple reductive dechlorinating cultures that are currently used for bioremediation *in situ*. The cultures used in these batch experiments (KB-1, WBC-2, and ACT-3) are dominated by *Dehalococcoides, Dehalobacter,* and *Dehalogenimonas,* respectively, which are commonly used for bioaugmentation in the field. Because these cultures are in fact used for this purpose, the findings from these experiments are particularly important in that they suggest that Cl-NOM can be added when these cultures are used for bioremediation to increase the rate (and possibly the extent) of dechlorination, reducing the time needed to reach clean-up goals, and as a result, the cost of remediation. Specific concentrations that are needed to achieve this effect need to be verified for each of these consortia individually and the toxicity of Cl-NOM needs to be determined prior to its addition to the environment.

The picture is less clear for hydrolytic dechlorinators and their response to Cl-NOM amendment. No obligate HB were detected in the flow-through reactor to which no PCE and only low concentrations of Cl-NOM were added.

Geobacter and *Desulfitobacterium*, both facultative HB (i.e., they do not require chlorinated compounds to "breathe" but can "breathe" these compounds if present), grew during the period of good Cl-NOM dechlorination, while *Desulfuromonas*, another facultative HB that was present, did not grow, suggesting that Cl-NOM amendment selects for specific facultative HB but not obligate HB, which are likely to dechlorinate more efficiently and completely. Furthermore, analysis of several dechlorinating genes determined that both reductive dechlorinating genes and hydrolytic dechlorinating genes were present, with the number of hydrolytic dechlorinating genes increasing during periods of good Cl-NOM dechlorination and reductive dechlorinating genes decreasing during those same periods.

These results suggested that both HB and hydrolytic dechlorinating bacteria are likely to dechlorinate Cl-NOM, with obligate HB increasing their rates of PCE dechlorination in the presence of Cl-NOM. In these experiments, Cl-NOM alone was not able to enrich for obligate HB nor was it able to enrich for organisms capable of PCE dechlorination when seeded with uncontaminated sediment.

The project is spent out and it is anticipated that the project will finish early, likely by the end of 2022. Any remaining work on the project will be paid from the PI's discretionary funds.

Amendment Request (9/20/2022):
This formal amendment request is to seek approval for the movement of $5,939 from the "Equipment/Tools/Supplies" category to the "Personnel" category. As a result of the pandemic and its impact on the ability of the graduate and undergraduate students to work consistently over the grant period, we ended up needing to shift additional funds to support the current graduate student. This change will not impact the project outcomes or timeline. Any additional supplies that need to be purchased will be funded from the PI's discretionary funds.

Amendment Approved by LCCMR 9/28/2022

Overall Project Outcome and Results:
Over half of the contaminated sites in Minnesota contain chlorinated pollutants—chemicals that contain chlorine atoms attached to a carbon framework—that can cause serious health effects. Once the chlorine atoms are removed, the carbon framework is non-toxic. Bacteria exist that can remove the chlorine atom from this carbon framework. One kind of bacteria "breathes" these compounds by removing the chlorine atom from the carbon framework (so-called halorespiring bacteria (HB)). As a result, they require the presence of chlorinated compounds to survive. Other bacteria (non-respiratory dechlorinators (NRD)) remove the chlorine atom to use the carbon framework for growth. Little is known about them. Natural chlorinated compounds (not pollutants) also exist as a natural part of soil (called chlorinated natural organic matter, or Cl-NOM).

In this research project the goals were to determine whether and how Cl-NOM stimulated dechlorination of pollutants. We found that Cl-NOM addition to HB led to the faster and more complete dechlorination of the common pollutant perchloroethylene (PCE). Currently, engineers actually add similar bacteria to the ones we tested to contaminated sites to improve degradation. Our research suggested that to further improve the degradation of these pollutants, Cl-NOM could be added with HB to increase the rate (and possibly the extent) of chlorine removal, reducing the time and cost to reach clean-up goals. We found that NRD are widespread and can dechlorinate chlorinated contaminants and grow as a result, potentially giving them a competitive advantage in nutrient-limited environments. Nevertheless, though Cl-NOM amendment selected for some HB and NRD, these organisms did not necessarily have the ability to dechlorinate the most common pollutants. One graduate student focused on this research for her Master's thesis (submitted to LCCMR) and another postdoctoral researcher published one peer-reviewed manuscript supported in part by this research (also submitted to LCCMR).

III. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1 Title: Determine how different amendments of natural compounds improve dechlorination

Description:
Experiments will be performed with the common pollutant trichloroethene (TCE) and sediment from contaminated and uncontaminated sites containing different initial amounts of TCE.

ACTIVITY 1 ENRTF BUDGET: **ENRTF Budget:** **$70,000**

Outcome	Completion Date
*1. Measure the dechlorination of PCE and TCE in sediments with **high ratios** of soil-based carbon to natural chlorinated compounds when amended with stimulants of varying ratios of soil-based carbon to natural chlorinated compounds*	*6/30/21*
*2. Measure the dechlorination of PCE and TCE in sediments with **low ratios** of soil-based carbon to natural chlorinated compounds when amended with stimulants of varying ratios of soil-based carbon to natural chlorinated compounds*	*6/30/21*

First Update March 1, 2020
Contaminated aquifer material has been obtained with which to begin experiments this summer. The material has been sub-sampled for organic carbon and chemical constituents such as nitrogen species and pH. Method development for measuring PCE, TCE, and their daughter products is underway, as is development of protocols for determining carbon content in experimental samples. A reactor has been constructed that will allow for the continuous culture of dechlorinating bacteria in the presence of controlled quantities of NOM and/or Cl-NOM. Effluent will be collected from the reactor over time and tested for TCE dechlorination activity.

Second Update September 1, 2020
The student has developed methods for measuring PCE, TCE and daughter products as well as chloride. She has generated NOM and Cl-NOM from the aquifer materials obtained previously. Feeding of the continuous flow reactor with Cl-NOM started and the reactor is being monitored with time for dechlorination of the Cl-NOM with samples taken for later evaluation of the genes and bacteria present. Initial dechlorination experiments have recently been set up.

Third Update March 1, 2021
Additional small-scale dechlorination experiments have been set-up to monitor for dechlorination as well as the generation of daughter products with two different aquifer materials and different quantities of amended NOM, Cl-NOM, and PCE. Additional NOM and Cl-NOM has been produced for use in experiments. Feeding of the continuous flow reactor has continued. Samples are taken weekly to monitor dechlorination via chloride production and biomass samples are also taken weekly. After an initial period of active Cl-NOM dechlorination in the reactor, dechlorination ceased. The reactor has been re-seeded with additional soil and higher concentrations of Cl-NOM to determine if that will stimulate dechlorination.

Fourth Update September 1, 2021
We have struggled in the last project period with establishing a robust PCE-dechlorinating culture. We have recently been in contact with a colleague who will send us a mixture of three active PCE-dechlorinating cultures. These cultures are expected to arrive by the end of October, 2021, at which point they will be used in experiments. In preparation for these experiments, the student working on the project has produced additional NOM and Cl-NOM. The student has also been continuing to operate the flow-through reactor, adding higher quantities of Cl-NOM to the reactor so that she can monitor the microbial response and the generation of chloride as a result of dechlorination of Cl-NOM. The addition of higher concentrations of Cl-NOM did stimulate

chloride production in the reactor, indicating that Cl-NOM dechlorination is active. Samples have been taken for biomass analysis and the DNA from these samples has been extracted.

Fifth Update March 1, 2022

Experiments were performed with the new dechlorinating culture. PCE was added and degraded within 2 weeks. DNA was collected from different time points of this experiment and will be extracted. Once extracted, analyses will be performed to determine how the numbers of the specific dechlorinating bacteria present changed over time. The experiment is currently being repeated with the addition of chlorinated NOM and NOM at different ratios (high NOM:low Cl-NOM and low NOM:high Cl-NOM) to determine the impact of those additions on PCE dechlorination. Initial data suggested that the PCE degraded rapidly in the presence of chlorinated NOM and NOM. Again, the populations that change in number over the degradation process will be determined. DNA will be analyzed for *Dehalococcoides*, *Dehalobacter* and *Dehalogenimonas* species, all of which should be present in the culture and could be involved in the dechlorination of PCE and could be stimulated by the amendment of NOM and/or Cl-NOM.

Update as of June 30, 2022

Project extended to June 30, 2023 by LCCMR 6/30/22 as a result of M.L. 2022, Chp.94, Sec. 2, Subd. 19, legislative extension criteria being met.

Sixth Update September 1, 2022

Experiments were performed to monitor the PCE dechlorination of obligate halorespiring bacteria (HB) in the absence of NOM and Cl-NOM ("no Cl-NOM"), in the presence of high concentrations of NOM plus low concentrations of Cl-NOM ("low Cl-NOM"), and in the presence of low concentrations of NOM plus high concentrations of Cl-NOM ("high Cl-NOM"). Results showed that when obligate HB are amended with Cl-NOM, dechlorination of PCE is faster and formation of the PCE daughter product, *cis*-DCE, occurs. When amended with a higher concentration of Cl-NOM, PCE dechlorination is faster compared to when cultures are amended with low concentrations of Cl-NOM. In the no Cl-NOM reactors, no *cis*-DCE was detected, suggesting that dechlorination was less complete. This is shown below in Table 1 where dechlorination rates for the no Cl-NOM experiments are compared to experiments with high and low Cl-NOM added. From these results, we can see that the addition of Cl-NOM accelerates the rate of dechlorination of PCE.

Table 1. Comparison between first order dechlorination rates and apparent chloride released per change in log copy number of HB for experiments with and without Cl-NOM and NOM during the reductive dechlorination of PCE.

Amendment	**First-order dechlorination rate (hr^{-1})**	**Apparent [Cl^-] released/Change in log(copy# of total bacteria) (mM/△log(copy#)**	**Apparent [Cl^-] released/Change in log(copy# of HB) (mM/$\Sigma_{\triangle log(copy\#)}$)**
D: no Cl-NOM or NOM	0.027 ± 0.01	0.212 ± 0.028	0.085 ± 0.011
A: low Cl-NOM, high NOM	0.038 ± 0.01	0.247 ± 0.027	0.125 ± 0.011
B: high Cl-NOM, low NOM	0.054 ± 0.009	0.188 ± 0.021	0.079 ± 0.008

These results suggest that addition of Cl-NOM at higher volumes to known obligate HB should be beneficial to dechlorination, though the quantity of Cl-NOM that should be amended to see this benefit is not known.

In the flow-through reactor, Cl-NOM was dechlorinated (Figure 1), though the dechlorination was not consistent, with periods of good dechlorination (high concentrations of Cl^- released) and poor dechlorination (low concentrations of Cl^- released).

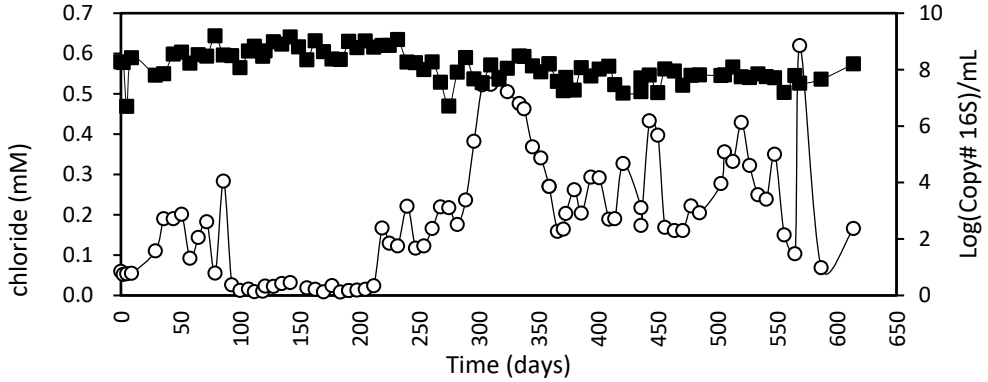


Figure 1. The concentration of chloride (○) is shown over time as result of dechlorination of chlorinated natural organic matter (Cl-NOM) for the same time series as the total number of Bacterial 16S rRNA genes (■) during the operation of the continuous flow reactor (CFR).

The reactor effluent never showed the ability to dechlorinate PCE, demonstrating that PCE-dechlorinating bacteria could not be enriched from uncontaminated sediment on only low concentrations of Cl-NOM. This suggests that although the amendment of Cl-NOM is promising as a way to enhance dechlorination, this amendment is most likely to be successful if added to a culture that is already capable of dechlorinating PCE.

Final Report Summary:
As stated above, obligate HB, in fact, the same organisms used in remediation efforts for bioaugmentation, are able to dechlorinate PCE faster and more completely when amended with Cl-NOM. When amended with a higher concentration of Cl-NOM, PCE dechlorination is faster compared to when cultures are amended with low concentrations of Cl-NOM. The addition of Cl-NOM accelerates the rate of dechlorination of PCE by these organisms, though it is still not clear the level of Cl-NOM amendment that is needed to achieve this result.

In the flow-through reactor, Cl-NOM was dechlorinated, though the dechlorination was not consistent, with periods of good dechlorination (high concentrations of Cl^- released) and poor dechlorination (low concentrations of Cl^- released). The reactor effluent never showed the ability to dechlorinate PCE, demonstrating that PCE-dechlorinating bacteria could not be enriched from uncontaminated sediment on only low concentrations of Cl-NOM. This suggests that although the amendment of Cl-NOM is promising as a way to enhance dechlorination, this amendment is most likely to be successful only if added to a culture that is already capable of dechlorinating PCE.

Additional research focused on a dechlorinating organism that is found in uncontaminated locations. These organisms were capable of dechlorinating relatively simple chlorinated pollutants (chloroacetate) and were also able to grow on the carbon present in that pollutant and incorporate it into their biomass. This is promising and suggests that if needed, such as in locations with low carbon concentrations, these non-respiratory dechlorinators can dechlorinate some simple pollutants to obtain an advantage over other competing bacteria. Nevertheless, their ability to dechlorinate more complex pollutants remains to be determined.

ACTIVITY 2 Title: Determine how the different groups of dechlorinating bacteria ("halorespiring" and "non-respiratory dechlorinators") are affected by these amendments

Description:
Genes are the codes that "tell" organisms which functions to perform (such as breathing chlorinated compounds). By analyzing genes, we can understand which organisms dominate (and by what mechanism) in a given sample. Samples will be taken from the experiments described above and the genetic material will be

extracted and analyzed over time. From this we will learn which genes are stimulated by the different amendments, which genes are responsible for different patterns of dechlorination, and which genes are initially present in different types of starting materials. By understanding how to "read" the genes used to dechlorinate pollutants, we will know the best amendment to add to stimulate dechlorination at a site without having to perform labor-intensive and expensive experiments.

ACTIVITY 2 ENRTF BUDGET: **ENRTF Budget:** **$80,000**

Outcome	Completion Date
1. Analyze the genes in initial starting material for the experiments	*1/31/20*
2. Analyze the types and quantities of genes in the experiments described in Activity 1 over time	*5/1/22*

First Update March 1, 2020
DNA has been extracted from the aquifer material to be used in experiments. The development of protocols for determining the microbial community composition and the number of target genes in a given sample is underway. As mentioned above, a reactor has been constructed that will allow for the continuous culture of dechlorinating bacteria in the presence of controlled quantities of NOM and/or Cl-NOM. Effluent will be collected from the reactor over time, as described above, and the numbers of target dechlorinating bacteria and genes associated with dechlorination will be measured.

Second Update September 1, 2020
Biomass samples are being collected weekly from the continuous flow reactor and will be processed to determine the numbers of dechlorinating bacteria present and genes associated with the measured dechlorination activity. Method development for analyzing the microbial community and dechlorination is continuing.

Third Update March 1, 2021
DNA was extracted from the biomass samples taken from the continuous flow reactor over the period of time prior to the commencement of dechlorination, during dechlorination, and after dechlorination stopped. The DNA was submitted for sequencing and results are expected in the next few weeks. These sequences will be analyzed to determine if the presence/growth/dynamics of particular organisms can be statistically linked to the dechlorination activity observed in the reactor. These samples will also be analyzed for the presence and quantity of targeted dechlorinating genes based on prior research. Results will be used in the future to identify additional target organisms/genes for analysis in the small-scale dechlorination experiments as well. The student performing this research has been undergoing training for analysis of the extremely large data sets that are produced during DNA sequencing.

Fourth Update September 1, 2021
The student has been analyzing the DNA sequencing data generated to date from the flow-through reactor and is currently generating taxonomy data for the reactor over time and as a function of chloride evolution (e.g., dechlorination of Cl-NOM). Taxonomy data is information regarding the genus and species of all the organisms present in the reactor; because of our past work on dechlorinating organisms and the rich databases that exist regarding the taxonomy of known dechlorinating bacteria, this data will be useful for identifying populations that grow in response to Cl-NOM addition and its dechlorination. The student working on the project has also determined which organisms and dechlorinating genes she would like to assess quantitatively in the samples taken from the flow-through reactor over time via quantitative polymerase chain reaction (qPCR) with specific primers and standards. She has ordered supplies to perform this quantitative analysis. These same supplies will be used to monitor the identified populations and genes in the PCE-dechlorinating culture experiments that will be performed with NOM and Cl-NOM addition.

Fifth Update March 1, 2022
Samples taken from the continuous flow reactor fed chlorinated NOM have been processed to extract DNA from the samples and have been submitted for sequencing. Samples from the period of reactor operation between February, 2020 (start-up) to August of 2021 have been sequenced and are currently being analyzed. These results should enable identification of both the populations that grew in response to Cl-NOM addition as well as the genes used for dechlorination. Preliminary results show that Geobacter and Desulfuromonas species were present in samples, both of which are linked to dechlorination. Statistical analysis will be performed to determine which organisms correlated to periods of greater dechlorination. In addition, the quantification (via quantitative polymerase chain reaction, or qPCR) of known genes, both those that code for the enzymes used in different dechlorination processes and those linked to particular known dechlorinating organisms will also be performed. These primers have been used in our laboratory for quantifying genes used for hydrolytic and reductive dechlorination--two different processes that benefit from different environmental conditions.

Update as of June 30, 2022
Project extended to June 30, 2023 by LCCMR 6/30/22 as a result of M.L. 2022, Chp.94, Sec. 2, Subd. 19, legislative extension criteria being met.

Sixth Update September 1, 2022
In reactors containing obligate HB dechlorinating PCE, PCE dechlorination occurred with the concomitant growth of Bacteria and the obligate HB *Dehalococcoides, Dehalogenimonas,* and *Dehalobacter.* In fact, the differences in initial and final concentrations of all organisms were statistically significant ($P<0.05$). This suggests that these HB were able to use PCE as an electron acceptor for growth. The number of bacteria and HB in the different treatments were different when comparing the no Cl-NOM and the low Cl-NOM treatments, with the low Cl-NOM treatments having more HB and bacteria. It is clear that obligate HB grew on PCE, but not clear whether they grew on Cl-NOM because the apparent quantity of Cl- released per change in the numbers of HB present was essentially the same in the no Cl-NOM and the high Cl-NOM treatments (Table 1).

With respect to the flow-through reactor, the total number of 16S rRNA genes for Bacteria decreased significantly during the good dechlorination period ($P<0.05$). This could be because Cl-NOM is toxic or inhibitory to some bacteria, though more work is needed to better understand this decrease. Nevertheless, when looking specifically at the numbers of the facultative HB *Geobacter, Desulfuromonas and Desulfitobacterium,* both *Geobacter* ($P = 0.002$) and *Desulfitobacterium* ($P = 0.002$) increased in number when dechlorination was better. This suggests that during periods of good dechlorination, both *Geobacter* and *Desulfitobacterium* were enriched, while *Desulfuromonas* was not enriched. This could indicate that these bacteria can dechlorinate Cl-NOM and use it as a (dechlorinated) electron donor when no other electron donor is present or perhaps, as an alternative electron acceptor during facultative organohalide respiration. Since *Geobacter* has substrate versatility and can utilize multiple electron donors, such as acetate, pyruvate, and hydrogen, and electron acceptors, such as PCE, nitrate, and fumarate, either possibility is reasonable. More work is needed, therefore, to determine what role, if any, *Geobacter* plays in the dechlorination of Cl-NOM. Increases in copy numbers of *Geobacter* and *Desulfitobacterium* could also be attributed to the NOM present in the reactor as well, which would have invariably been added when the Cl-NOM was added.

We also measured the concentrations of a reductive dehalogenase gene (rdh) and a hydrolytic dehalogenase gene (dh) found in the sediment used to seed the flow-through reactor and linked to dechlorination by previous research in our lab. Rdhs and dhs were present in all samples taken from the reactor. A paired t-test was performed to understand the significance between rdh and dh genes over the course of reactor operation. Results showed that rdhs were significantly higher than dhs ($P<0.05$). In addition, rdh genes were lower ($P = 0.0004$) during the good dechlorination phase while dh genes were higher during this phase ($P = 0.0262$). This could indicate that dechlorinators with dh genes are enriched during good dechlorination of Cl-NOM or that Cl-NOM selects for bacteria with dh genes over rdh genes. Finally, sequencing results showed that no obvious

obligate HB were present in this flow-through reactor, which again suggests that under these conditions, facultative HB and hydrolytic dechlorinators were likely active, rather than obligate HB.

Final Report Summary:
As stated above, in reactors containing PCE, PCE dechlorination occurred simultaneously to growth of the obligate HB *Dehalococcoides, Dehalogenimonas,* and *Dehalobacter*. The genes identifying these organisms may therefore be a good indicator that the system could be stimulated with Cl-NOM to dechlorinate pollutants. The number of bacteria and HB in the different treatments were different when comparing the no Cl-NOM and the low Cl-NOM treatments, with the low Cl-NOM treatments having more HB. It is clear that obligate HB grew on PCE, but it was not clear whether they grew on Cl-NOM.

With respect to the flow-through reactor, the total number of indicator genes for general *Bacteria* decreased significantly during periods of good dechlorination ($P<0.05$). This could be because Cl-NOM is toxic or inhibitory to some bacteria, though more work is needed to better understand this decrease. When looking specifically at the numbers of the facultative HB *Geobacter, Desulfuromonas* and *Desulfitobacterium,* both *Geobacter* ($P = 0.002$) and *Desulfitobacterium* ($P = 0.002$) increased in number when dechlorination was better. This suggests that during periods of good dechlorination, both *Geobacter* and *Desulfitobacterium* were enriched, while *Desulfuromonas* was not enriched. This could indicate that these bacteria can dechlorinate Cl-NOM and use it as a source of carbon and energy, or perhaps, as an alternative electron acceptor (something to "breathe") during facultative halorespiration. Since *Geobacter* has substrate versatility and can utilize multiple energy sources, such as acetate, pyruvate, and hydrogen, and electron acceptors, such as PCE, nitrate, and fumarate, either possibility is reasonable. Although the genes identifying *Geobacter* and *Desulfitobacterium* did increase in number when Cl-NOM dechlorination increased, the presence of these genes could not be linked to the dechlorination of pollutants. As a result, these genes were not thought to be particularly useful indicators of the potential for dechlorination.

We also measured the concentrations of a reductive dehalogenase gene (rdh) and a hydrolytic dehalogenase gene (dh) found in the sediment used to seed the flow-through reactor and linked to dechlorination by previous research in our lab. Rdhs and dhs were present in all samples taken from the reactor. A paired t-test was performed to understand the significance between rdh and dh genes over the course of reactor operation. Results showed that rdhs were significantly higher than dhs ($P<0.05$). In addition, rdh genes were lower ($P = 0.0004$) during the good dechlorination phase while dh genes were higher during this phase ($P = 0.0262$). This could indicate that dechlorinators with dh genes are enriched during good dechlorination of Cl-NOM or that Cl-NOM selects for bacteria with dh genes over rdh genes. Finally, sequencing results showed that no obvious obligate HB were present in this flow-through reactor, which again suggests that under these conditions, facultative HB and hydrolytic dechlorinators were likely active, rather than obligate HB. Again, the presence of these genes could not be linked to the dechlorination of pollutants. Therefore, these genes were not thought to be particularly useful indicators of the potential for dechlorination.

IV. DISSEMINATION:

Description:
The target audience for results from this research will be professionals in the area of hazardous waste treatment. Specific targets will be environmental engineers and scientists in academia, industry, state agencies such as the MPCA, and environmental consultants. Results will be disseminated through scholarly publications in peer-reviewed journals such as *Environmental Science and Technology*. Results from the research project will also be presented at regional conferences such as the *Minnesota Water* conference.

The Minnesota Environment and Natural Resources Trust Fund (ENRTF) will be acknowledged through use of the trust fund logo or attribution language on project print and electronic media, publications, signage, and other communications per the ENRTF Acknowledgement Guidelines.

First Update March 1, 2020
No dissemination efforts have been made, as the project is not advanced enough at this point.

Second Update September 1, 2020
No dissemination efforts have been made at this point.

Third Update March 1, 2021
No dissemination efforts have been made at this point.

Fourth Update September 1, 2021
The student working on the project presented her results to data in May, 2021 at a seminar at the University of Minnesota. No additional dissemination efforts have been made at this point.

Fifth Update March 1, 2022
No additional dissemination efforts have been made. The student working on the project will present an update of her research at the University of Minnesota in April, 2022.

Update as of June 30, 2022
Project extended to June 30, 2023 by LCCMR 6/30/22 as a result of M.L. 2022, Chp.94, Sec. 2, Subd. 19, legislative extension criteria being met.

Sixth Update September 1, 2022
The student working on the project presented her results to data in April, 2022 at a seminar at the University of Minnesota. She also presented her data at a conference in May, 2022.

Final Report Summary:
One paper has been supported in part with this research (listed below and sent to LCCMR with the final report). Multiple presentations about the research have been given at conferences. The student supported by this funding for her Master's research is now a practicing engineer for a large regional firm, and as such is able to further disseminate these results.

Bhattarai, S., Temme, H., Jain, A., Badalamenti, J. P., Gralnick, J. A., Novak, P. J. (2022). The Potential for Bacteria from Carbon-Limited Deep Terrestrial Environments to Participate in Chlorine Cycling. *FEMS Microbiology Ecology*. 98:1-11.

V. ADDITIONAL BUDGET INFORMATION:

A. Personnel and Capital Expenditures

Explanation of Capital Expenditures Greater Than $5,000: N/A

Explanation of Use of Classified Staff: N/A

Total Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation:

Enter Total Estimated Personnel Hours for entire duration of project: 2,160	Divide total personnel hours by 2,080 hours in 1 yr = TOTAL FTE: 0.35 FTE/yr

Total Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation:

Enter Total Estimated Contract Personnel Hours for entire duration of project: N/A	Divide total contract hours by 2,080 hours in 1 yr = TOTAL FTE: N/A

VI. PROJECT PARTNERS:

A. Partners outside of project manager's organization receiving ENRTF funding
None

B. Partners outside of project manager's organization NOT receiving ENRTF funding
None

VII. LONG-TERM- IMPLEMENTATION AND FUNDING:
Minnesota has impressive environmental resources but also a large number of sites that need to be remediated at a large cost. Novak has worked on the halorespiration of chlorinated pollutants for about 20 years. She is the first to perform research on the existence of halorespiring bacteria in uncontaminated environments and the first to show that pollutant degradation can be stimulated through the addition of uncontaminated soil extracts to the bacteria present. The goal of this project is to identify how the organisms that naturally cycle chlorine in uncontaminated Minnesota environments can best be deployed to detoxify chlorinated pollutants. This research should enable the development of new remediation technologies that are more effective and less expensive than those currently used, cleaning more sites and improving Minnesota's environment.

VIII. REPORTING REQUIREMENTS:

- Project status update reports will be submitted March 1 and September 1 each year of the project
- A final report and associated products will be submitted between June 30 and August 15, 2022

IX. SEE ADDITIONAL WORK PLAN COMPONENTS:

A. Budget Spreadsheet
B. Visual Component or Map
C. Parcel List Spreadsheet: N/A
D. Acquisition, Easements, and Restoration Requirements: N/A
E. Research Addendum

Attachment A:
Environment and Natural Resources Trust Fund
M.L. 2019 Project Budget -Final
Legal Citation: M.L. 2019, First Special Session, Chp. 4, Art. 2, Sec. 2, Subd. 04s
Project Manager: Paige J. Novak
Project Title: Stimulating bacteria to degrade chlorinated industrial contaminants
Organization: University of Minnesota
Project Budget: $150,000
Project Length and Completion Date: 4 years, June 30, 2023
Date of Report: January 3, 2023

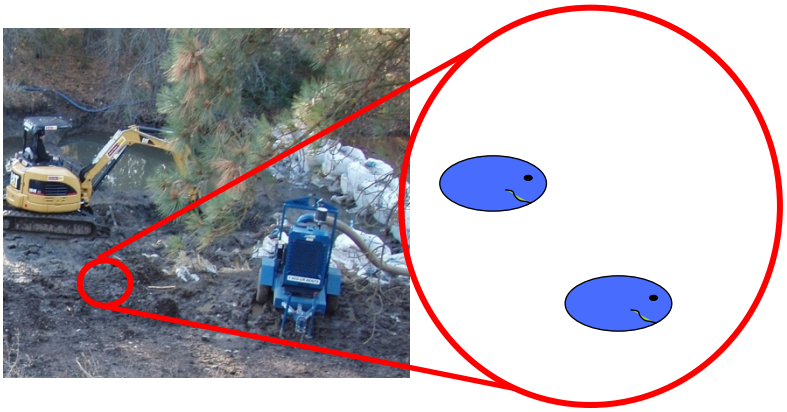
ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Budget (9/28/22)	Amount Spent	Balance
BUDGET ITEM			
Personnel (Wages and Benefits)	$ 124,291	$ 124,291	$ -
Novak (PI, 1% time per year for three years, salary 75% of cost, fringe benefits 25% of cost). Project supervision, provide guidance experimental design and sample analysis. Total estimated cost is $11,136. Graduate student (33% time per year for three years, 57% salary, 32% tuition, 11% fringe benefits). Conducting laboratory experiments and analyzing samples using chemical and genetic techniques. Undergraduate student ($5,000 estimated) to assist the graduate student with basic reactor operation and sample analysis. Total estimated cost of both graduate and undergraduate students is $95,216.			
Professional/Technical/Service Contracts			
		$ -	$ -
Equipment/Tools/Supplies			
Funds for laboratory supplies are requested ($11,000/year). This includes, but is not limited to: DNA soil extraction kits, materials for quantifying genes present, primers for deep genetic sequencing, pipette tips, eppendorf tubes, glassware, chemicals for standards and experiments, analytical consumables, analytical fees, solvents, reagents, and gloves. Funds ($8,000 total) are also requested for sequencing via Illumina sequencing. Additional funds budgeted for equipment repair and maintenance ($2,648).	$ 25,709	$ 25,709	$ -
	$ -	$ -	
COLUMN TOTAL	$ 150,000	$ 150,000	$ -

OTHER FUNDS CONTRIBUTED TO THE PROJECT	Status (secured or pending)		Spent	Balance
Non-State:			$ -	$ -
State:			$ -	$ -
In kind: Novak will provide unpaid time to the project (including 2% cost-share). Because the project is overhead-free, laboratory space, electricity, and other overhead costs are provided in kind. The University of Minnesota overhead rate is 54%.			$ -	$ -

PAST AND CURRENT ENRTF APPROPRIATIONS	Amount legally obligated but not yet spent		Spent	Balance
Current appropriation:			$ -	$ -
Past appropriations:			$ -	$ -

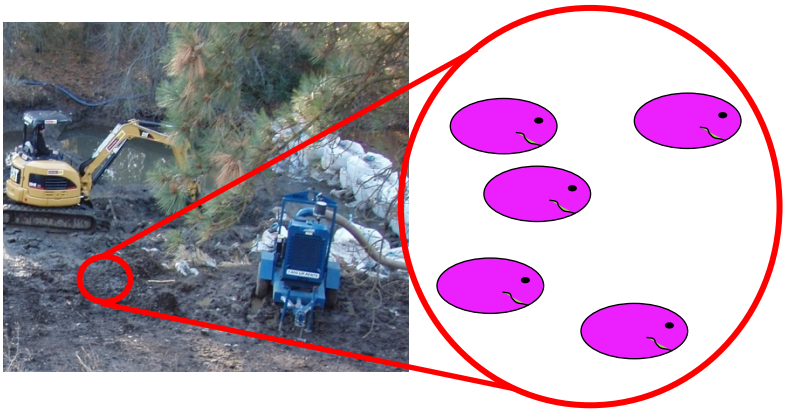
Stimulating bacteria to degrade chlorinated industrial contaminants

The problem



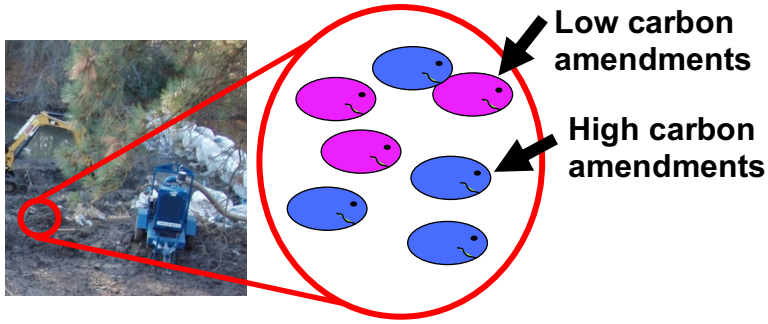
- Bacteria exist that "breathe" chlorinated contaminants (**blue bacteria**)
- Bacteria need higher concentrations of contaminants to thrive
- There is often too little contamination to support the bacteria, but too much to consider the site safe

A potential solution



- Other bacteria exist that dechlorinate compounds but don't "breathe" these compounds (**pink bacteria**)
- They work more slowly, but may degrade the contaminants to lower concentrations, cleaning the site to a greater extent

What we need to know



- How do we best stimulate both types of bacteria for fast and complete dechlorination?
- How can we monitor their progress without expensive and time-consuming experiments?